In vivo analysis of Pseudomonas Auriginosa Bacteria and the effects of a novel bacterial molecule by whole cell High Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy

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Introduction– *Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium that is capable of surviving in different natural environments, although it is best known as a multi-antibiotic resistant human pathogen associated with hospital acquired infections (1, 2) and is a major cause of morbidity and mortality in cystic fibrosis (CF) patients. Cell High Resolution Magic Angle Spinning (HRMAS) Nuclear Magnetic Resonance (NMR) spectroscopy has gained recent popularity. HRMAS is a novel non-destructive technique that substantially improves spectral line-widths and allows high-resolution spectra to be obtained from intact cells, cell culture tissues, and unprocessed tissue. HRMAS ^{1}H-MRS has enabled us to investigate relationships between metabolites and cellular processes. To avoid any spontaneous modification by the extraction/ purification of the metabolites, we used HRMAS-NMR to investigate live *Pseudomonas aeruginosa* with or without exogenous addition of the low molecular weight molecule, 2-amino acetophenone (2-AA), produced and excreted by PA to determine any changes in their metabolic profile, in order to characterize the metabolic profile of the cells and the cell surface structure. We were interested in 2-AA, because 2-AA reduces bacterial pathogenicity in vivo in flies and in an acute mouse infection model. Such reduction appears to be linked with 2-AA's ability to promote bacterial phenotypic changes associated with chronic infections. 1-dimensional and 2-dimensional 1H HRMAS NMR were use.

Materials and Methods– Cells Samples. The P. aeruginosa strain used in this study was RifR human clinical isolate UCBPP-PA14. The mutants of PA14 described in the paper are isogenic to UCBPP-PA14. The bacteria were grown at 370C on Luria-Bertini (LB) broth or on plates of LB agar containing appropriate antibiotics or the compounds unless mentioned otherwise. The overnight PA14 cultures were grown in LB and diluted the following day in fresh media in triplicates. 10ml of culture at OD 600nm 2.0 was centrifuged and the pellet washed once with PBS.NMR analyses. Bacterial samples (50ul and 40ul) were introduced into the 4mm Zr rotor and 10 µl D2O (deuterium lock reference) containing 10 mM TSP (trimethylsilyl propionic-2,2,3,3-d4 acid, Mw=172, δ=0ppm, external chemical shift reference) was added to the rotor with the sample. 1H HR-MAS NMR experiments were performed on a Bruker Bio-Spin Avance NMR spectrometer (600.13 MH) using a 4mm triple resonance (^{1}H, ^{13}C, ^{2}H) HRMAS probe (Bruker). The temperature was controlled at 4°C by a BTO-2000 unit in combination with a MAS pneumatic unit (Bruker). Samples were spun at 3000 Hz and two different types of one dimensional (1D) proton spectra were acquired by using a water-suppressed spin-echo Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [90-(τ-180-τ)n-acqu] .Further investigation for metabolites was performed using a novel approach that combines a two-dimensional (2D), solid-state, HR-MAS proton (1H) NMR method, TOBSY (TOtal Through-Bond SpectroscopY), which maximizes the advantages of HRMAS and a robust classification strategy (3).

Results– NMR analysis was carried out on strain PA14. We identified from the combined analysis of 1D and 2D spectra 25 metabolites in *P. aeruginosa*. A representative 1D 1H CPMG HRMAS NMR spectrum obtained from live bacterial cells of PA14 strain are reported in Figure 2. This first approach to cells analysis is fast and provide important information. However, there are peaks overlap in the 1D, and it is thus difficult to assign peaks to specific molecules. Comparison between PA14 and PA14+2-AA are reported in figure Figure 3). We are able to identify the metabolic difference (Fig 3).

Discussion- A variety of informative metabolites were detected, (i.e., capsular polysaccharides structure, signals due to their N-Acetyl signal (2.02÷2.33/ 4.10÷4.33 ppm); these along with the phospholipids signal can be very informative about the consistency of bacterial membrane. Other metabolites like betaine compounds were detected. These molecules are well known to act as osmoprotectors and recently shown to preserve L-homoserine lactone (HSL) and 2-heptyl-4-quinolone (HHQ) bacterial cell-to-cell signaling molecules production. Betaine is synthesized from betaine aldehyde via the betB gene product. The ability of the bacterium to produce phospholipase C, we found the presence of PC, correlates with its virulence when infecting the lungs of the patients with cystic fibrosis suggesting that choline precursors are used and metabolized during infection. Use of both 1- and 2-dimensional ^{1}H HRMAS NMR results in a powerful technique in a variety of in vivo studies, including live bacterial cells in this study. Multidimensional HRMAS NMR using intact bacterial cells represents a promising method that could provide in vivo information of metabolomics in live bacteria. To this end, it can be complementary to existing chemical and biological methods. This technique may prove to be a helpful tool in gene function validation, the study of pathogenesis mechanisms, the classification of microbial strains into functional/clinical groups and the testing of anti-bacterial agents. Moreover the technique could be use to distinguish the metabolic profile of different mutant.

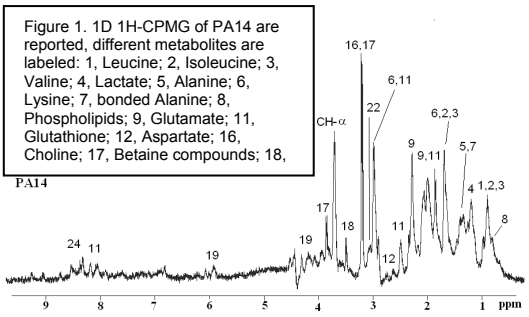


Figure 1. 1D 1H-CPMG of PA14 are reported, different metabolites are labeled: 1, Leucine; 2, Isoleucine; 3, Valine; 4, Lactate; 5, Alanine; 6, Lysine; 7, bonded Alanine; 8, Phospholipids; 9, Glutamate; 11, Glutathione; 12, Aspartate; 16, Choline; 17, Betaine compounds; 18,

Figure 2. TOBSY of PA14

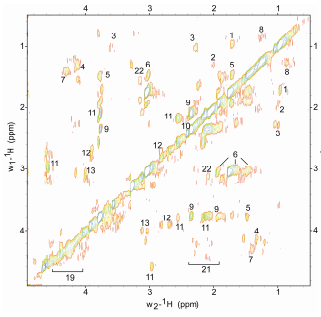
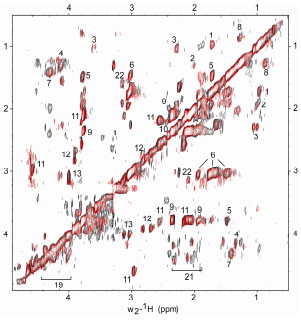


Figure 3. Super impose TOBSY of PA14 and 2AA



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